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Proteomic Insights into Metabolic Adaptations in *Alcanivorax borkumensis* Induced by Alkane Utilization

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***Alcanivorax borkumensis* is a ubiquitous marine petroleum oil-degrading bacterium with an unusual physiology specialized for alkane metabolism. This "hydrocarbonoclastic" bacterium degrades an exceptionally broad range of alkane hydrocarbons but few other substrates. The proteomic analysis presented here reveals metabolic features of the hydrocarbonoclastic lifestyle. Specifically, hexadecane-grown and pyruvate-grown cells differed in the expression of 97 cytoplasmic and membrane-associated proteins whose genes appeared to be components of 46 putative operon structures. Membrane proteins up-regulated in alkane-grown cells included three enzyme systems able to convert alkanes via terminal oxidation to fatty acids, namely, enzymes encoded by the well-known *alkB1* gene cluster and two new alkane hydroxylating systems, a P450 cytochrome monooxygenase and a putative flavin-binding monooxygenase, and enzymes mediating β-oxidation of fatty acids. Cytoplasmic proteins up-regulated in hexadecane-grown cells reflect a central metabolism based on a fatty acid diet, namely, enzymes of the glyoxylate bypass and of the gluconeogenesis pathway, able to provide key metabolic intermediates, like phosphoenolpyruvate, from fatty acids. They also include enzymes for synthesis of riboflavin and of unsaturated fatty acids and cardiolipin, which presumably reflect membrane restructuring required for membranes to adapt to perturbations induced by the massive influx of alkane oxidation enzymes. Ancillary functions up-regulated included the lipoprotein releasing system (Lol), presumably associated with biosurfactant release, and polyhydroxyalkanoate synthesis enzymes associated with carbon storage under conditions of carbon surfeit. The existence of three different alkane-oxidizing systems is consistent with the broad range of oil hydrocarbons degraded by *A. borkumensis* and its ecological success in oil-contaminated marine habitats.**

Alcanivorax borkumensis is a key marine oil-degrading bacterium that can dramatically increase in numbers after an oil spill and become the most abundant microbe in oil-polluted waters (21, 26, 27, 43). The list of sites where it has been isolated and shown to be involved in oil degradation grows with microbiological investigations of oil spills (9, 37; M. M. Yakimov, personal communication). The physiology of *A. borkumensis* is characterized by oligotrophy and a highly restricted growth substrate profile, namely, petroleum hydrocarbons plus a few organic acids, though the spectrum of hydrocarbons degraded is exceptionally broad. Its unusual metabolic features, presumed global importance in the natural biological removal of oil entering marine systems, and biotechnological potential for mitigation of the ecological damage caused by oil spills stimulated recent and current functional genomic studies of this organism. A key question concerning the ubiquity and competitive success of *A. borkumensis* in many marine locations is the genomic and biochemical basis of its physiological specialization and broad hydrocarbon substrate spectrum.

Hydrocarbon degradation is generally initiated by monooxygenases, encoded by *alkB* genes, which are widely present in oil-degrading gram-negative bacteria (41). Additional alkane hydroxylating systems, such as alkane hydroxylase systems belonging to the cytochrome P450 family, have been found in *Rhodococcus rhodochrous* ATCC 19067 (4) and *Acinetobacter calcoaceticus* EB104 (33). It has recently been shown that *A. borkumensis* possesses at least two *alkB*-like genes, namely, *alkB1* and *alkB2* (20, 46), both of which were induced by C_{14} alkanes in *A. borkumensis* strain AP1 (46). However, knockout mutagenesis revealed that only *alkB1* is essential for the degradation of C_6 alkanes in *A. borkumensis* strain SK2 (20). Neither single *alkB1* or *alkB2* knockout mutants nor a double mutant exhibited significant growth deficiencies on C_8 to C_{16} alkanes, so it was postulated that *A. borkumensis* specifies additional degradation systems for alkane degradation. Consistent with this conclusion are our own transposon mutagenesis experiments with *A. borkumensis* strain SK2, which yielded only alkane-defective mutants with mutations mapping in genes specifying metabolic steps downstream of the initial alkane oxidation step but not in *alkB1* and *alkB2* (data not shown). Thus, there is circumstantial, albeit negative, evidence for the existence of multiple systems for the primary oxidation of long-chain alkanes.

With the recently finished genome sequence of *A. borkumensis* SK2 (S. Schneiker et al., unpublished data), we sought evidence of potential additional alkane oxidation systems induced by growth of *A. borkumensis* on alkanes. We report here a proteomic study of differentially regulated proteins in the membrane and the cytoplasmic fractions of *A. borkumensis*

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strain SK2 and propose from the results obtained the nature of the alkane oxidation systems available and of the metabolic adaptations to growth on alkanes.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *A. borkumensis* strain SK2 (DSM 11573) was grown on ONR7a medium at 30°C with agitation with either 2% (wt/vol) pyruvate or 1.5% hexadecane as the carbon and energy source, as described earlier (50). In order to imitate the conditions of an oil spill in the environment (high carbon concentration and nitrogen limitation), we initially decided to create conditions of a high C/N ratio (100:1) by having either 1.5% (wt/vol) hexadecane or 2% pyruvate. One milliliter of a preculture was used to inoculate 200 ml of the medium. Growth was monitored by measuring the optical density at 600 nm. The cells were harvested in the early exponential phase at an optical density at 600 nm of 1.5 for the pyruvate culture and of 0.3 for the hexadecane culture.

Preparation and two-dimensional (2-D) gel electrophoresis of the cytoplasmic protein fraction. Cultures were harvested by centrifugation at 4°C at 8,000 × *g* for 15 min, resuspended, and washed twice in phosphate-buffered saline (38). The resulting pellet was stored at −20°C. For protein extraction, a cell pellet from a 200-ml culture was allowed to thaw on ice, and then 1 ml of rehydration buffer (4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 30 mM dithiothreitol, 20 mM Tris base, 7 M urea, 2 M thiourea, 0.2% immobilized pH gradient buffer, and one pellet of protease inhibitor cocktail [Complete Mini Boehringer, 20 ml^{-1}]) was added and the suspension sonicated on ice with a 3.5-mm sonication probe (Labsonic U; Braun, Melsungen, Germany) six times (91 W, repeating cycle of 0.6 s), with a 30-s interval between each cycle. The microcentrifuge tubes were centrifuged to remove the cell debris. Benzonase Nuclease (Novagen; 1/1,000 dilution) and $MgCl_2$ (2 mM final concentration) were added to remove nucleic acids, and the tubes were incubated at 4°C for 1 h, after which the extracts were transferred to polycarbonate centrifuge tubes (Beckmann) and centrifuged for 45 min at 4°C (ca. 30,000 × *g*; Rotor TLA100.3) (Beckmann ultracentrifuge). Two volumes of phenol (equilibrated with Tris-EDTA buffer, pH 7.4) and two volumes of water were added to one volume of sample, vortexed vigorously, incubated on ice for 15 min, and centrifuged. The aqueous phase was discarded without disturbing the white-protein-containing lower phase. Two volumes of water were added, and the procedure was repeated twice. Ice-cold acetone was added to precipitate proteins before the final centrifugation step. Protein pellets were washed twice with ice-cold acetone, air dried, and resuspended in up to 500 μl rehydration buffer. The total protein concentration was determined by the Bradford method, using bovine serum albumin as the standard (2).

Two-dimensional gel electrophoresis was carried out as previously described (18). Briefly, 500 μg of protein in a total volume of 300 μl was subjected to isoelectric focusing in IPG Ready Strips (17 cm; pH 3 to 6 or 4 to 7) (Bio-Rad, Munich, Germany). The gels were passively rehydrated for 2 h followed by an active rehydration step for 12 h at 50 V in rehydration buffer on a PROTEAN II Cell (Bio-Rad, Munich, Germany). Isoelectric focusing was performed at 5,000 V for 150 V · h. For the second dimension, the gels were soaked twice for 15 min in equilibration solution (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate [SDS], 50 mM Tris base, pH 8.8), the first time with 2% dithiothreitol and the second time with 2.5% iodoacetamide. The strips were then applied to 1.5-mm-thick gradient SDS-polyacrylamide (10% to 15% [wt/vol]) gradient gels, which were subjected to electrophoresis at 100 V in an IsoDalt system (Amersham Pharmacia Biotech, Uppsala, Sweden) overnight. Gels were stained with colloidal Coomassie brilliant blue dye (CBB G-250) and proteins were cut out of gels, destained, and prepared for matrix-assisted laser desorption ionization (MALDI)–time of flight analysis, according to Wissing et al. (49) and Hale et al. (19). The peptide mass fingerprints obtained were identified using an *A. borkumensis* SK2 protein database that was built up in conjunction with a genome-sequencing study.

Preparation and two-dimensional gel electrophoresis of the membrane fraction. Cells from a 400-ml culture of *A. borkumensis* grown at 30°C on ONR7a medium were harvested (4,500 × *g* for 20 min), resuspended in 10 ml 100 mM Tris-HCl (pH 7.0) buffer, incubated at 37°C in a shaking water bath for 3 h, and sonicated on ice for 20 min at 50% power and a duty cycle of 5 in a Branson Sonifier. Membranes were pelleted by centrifugation at 30,000 × *g* for 30 min at 4°C, washed twice with the buffer mentioned above, and stored at −70°C until use. Membrane proteins were isolated after resuspension of membranes in an equal volume of buffer, 100 mM Tris-HCl (pH 7.0), containing 2% sodium-lauryl sarcosinate, 150 mm NaCl, and incubation at 37°C for 1 h to facilitate inner membrane solubilization (14). Two volumes of equilibrated phenol (AppliChem GmbH, Darmstadt, Germany) were added to 1 volume of sample, and the suspension was vigorously vortexed, incubated on ice for 10 min, and centrifuged (16,000 × *g*, 15 min, 4°C). The top aqueous phase was discarded, 2 volumes of distilled water were added, and the mixture was vortexed, incubated on ice for 10 min, and centrifuged (4,000 × *g*, 15 min, 4°C). The aqueous phase was discarded and the step repeated. Then, 1 ml of ice-cold acetone was added and the tubes were inverted several times, incubated on ice for 10 min, and centrifuged (16,000 × *g*, 15 min, 4°C). The liquid phase was discarded and the remaining pellet air dried for 5 to 10 min. Pellets were suspended again in the solubilization solution and analyzed by two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis was carried out as described previously (42), although with small modifications. Briefly, approximately 200 μg of protein was applied to 24-cm, pH 3 to 10 NL IPG strips (ReadyStrip; Bio-Rad, CA) and fractionated by isoelectric focusing on a Protean IEF Cell (Bio-Rad) at a maximum voltage of 10,000 V for approximately 320 KV · h according to the following program: 50 V for 100 V · h, 300 V for 800 V · h, 600 V for 2,000 V · h, 2,500 V for 5,000 V · h, 7,500 V for 30,000 V · h, and 10,000 V until the end of run. The strips were then loaded on 1.5-mm-thick 10% to 15% gradient SDS-polyacrylamide gels and run overnight on a Hoefer DALT system (Amersham Biosciences). The gels were then fixed with 10% trichloroacetic acid and stained with Coomassie brilliant blue G250, and digitized images of stained gels were acquired by scanning. Protein spots were excised from preparative gels; in situ trypsin digestion (sequencing-grade modified trypsin; Promega, Madison, WI) and peptide extractions were performed as described previously (18). Peptide samples were eluted from ZipTips μ-C_{18} (Millipore, Bedford, MA), using 1.5 μl of saturated α-cyano-4-hydroxycinnamic acid (Sigma), and analyzed by protein sequence by using quadrupole time of flight mass spectrometry.

Prediction of putative promoters and operon structures. A prediction of putative operons encompassing genes of interest was made on the basis of close vicinity (less than 50 bp) of similarly orientated genes encoding predicted functionally related proteins (17) and/or whether a good putative promoter was found upstream of the first gene of the predicted operon. Putative σ^{70} (22)-, σ^{54} (24)-, σ^{38} (6)-, and σ^{32} (13)-dependent standard-type promoters of *Alcanivorax borkumensis* were identified by sequence homology to published consensus sequences. We did not search for promoters of other types, as most of the putative operons of interest were actually preceded by one of these, which, in the context of predicting putative operon structure, was considered sufficient.

Prediction of putative functions of novel proteins based on sequence homologies. The putative functions of proteins with little or no homology to known proteins were predicted using tools currently available on the World Wide Web. Firstly, the amino acid sequences of the identified proteins were obtained from the *Alcanivorax borkumensis* SK2 genome and subjected to a BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) homology search. Many of the proteins contained a conserved domain. Secondly, the amino acid sequences were also aligned to the *A. borkumensis* genome itself ("BLAST to self"). Thirdly, those genes/proteins with no functional domains were further analyzed by Pfam (http://www.sanger.ac.uk/Pfam/). Finally, the protein sequences were also checked for the presence of transmembrane domains using the program Tmpred (28).

RESULTS AND DISCUSSION

Proteins differentially expressed by cells grown on alkanes or pyruvate. Since initial 2-D gel profiles of *A. borkumensis* proteins on IPG strips from pH 3 to 10 revealed that most of the cytoplasmic proteins focused in the range of pH 4 to 7 (data not shown) and the median value of pI for all the putative proteins from the draft genome sequence of *A. borkumensis* was calculated to be around 6.0, we concentrated on the pI range of pH 4 to 7. Differentially expressed (up- or down-regulated or uniquely expressed, under either condition) cytoplasmic proteins influenced by alkane degradation by SK2 were identified by comparing two-dimensional gel electrophoresis images of proteins from cells in early stationary phase, growing with either pyruvate or hexadecane as the sole source of carbon and energy, and MALDI mass spectrometry analysis of differentially expressed proteins. Figure 1 shows Coomassie brilliant blue-stained 2-D gels of the cytoplasmic fraction of SK2 cells grown on either pyruvate (panel A) or hexadecane

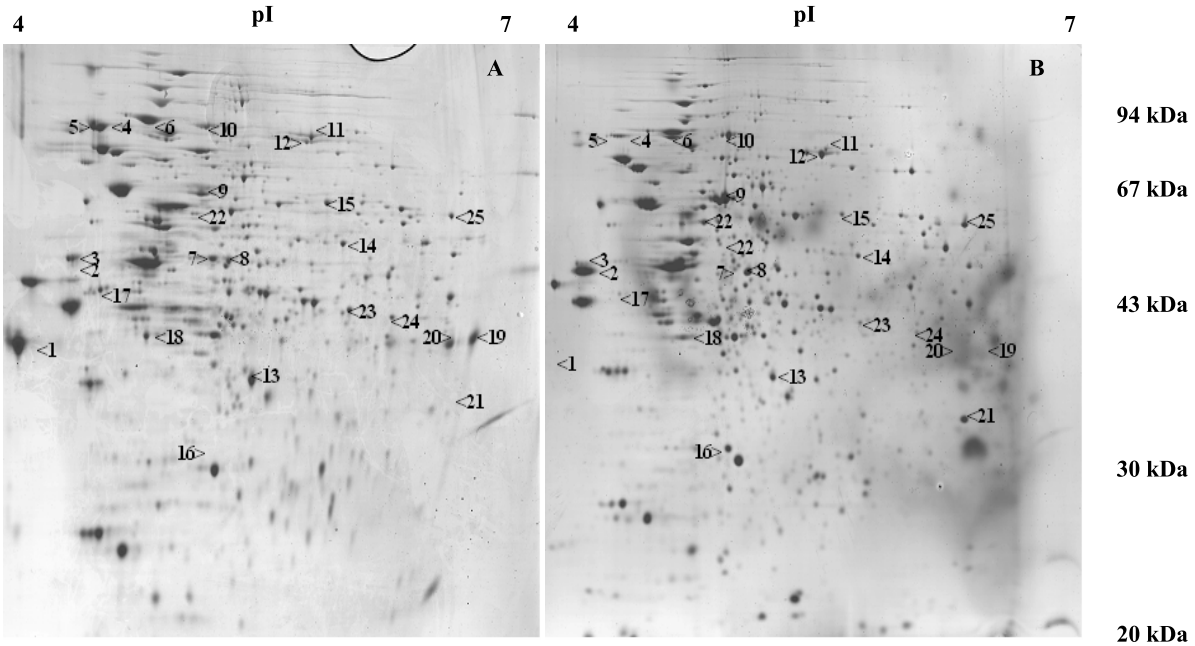

FIG. 1. A 2-D map of the cytoplasmic proteins of *Alcanivorax borkumensis* SK2. Cells were grown on either pyruvate (A) or hexadecane (B). Isoelectric focusing was performed using an IPG strip of pH 4 to 7. Proteins showing reproducible differential expression are numbered.

(panel B). Expression of most of the cytoplasmic proteins was found not to depend on the carbon source used, though 25 proteins consistently showed differential expression (Tables 1 and 2). Of these, 7 appeared exclusively in gels of extracts from hexadecane-grown cells, namely, outer membrane protein OprF (spot 2C [C, cytoplasmic; M, membrane]), phosphoenolpyruvate synthase PspA-1 (spot 6C), malic enzyme MaeB (spot 8C), isocitrate lyase AceA (spot 9C), 50S ribosomal protein RplY (spot 16C), 30S ribosomal protein RpsB (spot 21C) and a putative acyl coenzyme A (acyl-CoA) dehydrogenase (spot 22C), and 11 appeared exclusively in gels of extracts from pyruvate-grown cells, namely, outer membrane protein (spot 1C), outer membrane receptor FecA (spot 4C), fimbrial assembly protein precursor PilQ (spot 5C), DABA (2,4-diaminobutyrate) aminotransferase EctB (spot 7C), NADH-dependent isocitrate dehydrogenase Icd (spot 11C), conserved hypothetical protein (spot 17C), LysM domain protein (spot 18C), phosphate ABC transporter PstS (spots 19C and 20C), acyl-CoA dehydrogenase (spot 23C), and hypothetical protein (spot 24C). Three proteins showed significantly increased expression in alkane-grown cells, namely, malate synthase GlcB (spot 10C; 4×), fatty acid oxidation complex alpha subunit (spot 12C; 61×) and cytochrome P450 (spot 25C; 1.9×), while four others were up-regulated in pyruvate-grown cells: hypothetical protein (spot 3C; 2.7×), acetyl-CoA carboxylase AccA (spot 13C; 2.2×), acetyl-CoA carboxylase AccC (spot 14C; 1.9x), and long-fatty-acid CoA ligase FadD (spot 15C; 2.7×). Identical MALDI spectra were obtained for the sets of twin spots 17C/51C, 42C/44C, and 6C/83C.

We employed a wider pI range (2–7) for the membrane fraction to take into account the fact that most of the membrane proteins have rather extreme pIs. Obtaining clear 2-D images of membrane fractions from hexadecane-grown cells was problematic, probably due to the hydrophobic nature of the proteins expressed on hexadecane that affected isoelectric focusing. To circumvent the resulting difficulties in differentiating protein expression in pyruvate- and hexadecane-grown cells, we extracted all spots from both gels. Figure 2 shows Coomassie brilliant blue-stained 2-D gels of membrane proteins of *A. borkumensis* grown on pyruvate (panel A) or hexadecane (panel B), and Tables 1 and 2 list the differentially expressed proteins. Thirty-eight proteins were detected exclusively in hexadecane-grown cells, namely, alkane 1-monooxygenase AlkB (spot 1M), outer membrane lipoprotein LolB (spot 2M), rubredoxin AlkG (spot 4M), hypothetical proteins (spots 3M, 5M, and 7M to 9M), ABC transporters (spots 6M and 83M), outer membrane protein OprG (spot 13M), the AlkS regulator of the *alkB1GHJ* operon (spot 14M), aldehyde dehydrogenase AlkH (spot 15), alcohol dehydrogenase AlkJ (spot 16M), medium-chain fatty acid CoA ligase (spots 17M and 51M), succinate dehydrogenase SdhD (spot 22M), long-chain fatty acid CoA ligase FadB (spot 23M), fatty acid oxidation complex FadB2 (spot 26M), (*S*)-2-hydroxy fatty acid dehydrogenase RibD (spot 27M), permease protein (spot 29M), cardiolipin synthase (spot 30M), conserved hypothetical protein (spot 32M), 3-oxoacyl-(acyl-carrier-protein) synthase (spot 34M), fatty acid desaturase (spots 42M and 44M), poly-β-hydroxybutyrate polymerase PhaC (spot 45M), putative metabolite transport transmembrane protein (spot 49M), ABC transporters (spots 63M and 83M), putative membrane protein (spot 64M), multidrug/solvent RND membrane fusion protein (spot 67M), putative membrane-associated metalloprotease (spot 68M), putative lipoprotein (spot 73M), dihydroxy-acid dehydratase LlyD-1 (spot 75M), putative monooxygenase (spot 77M), alcohol dehydrogenase AlkJ-2 (spot 78M), nitrite extrusion protein NarK (spot 87M), and sodium solute transporter family protein (spot

TABLE 1. Differentially expressed proteins derived from both membrane and cytoplasmic protein fractions of *A. borkumensis* SK2 grown on either hexadecane or pyruvate as carbon source

Spot no.	pI	Mol wt	Gene function or functional category[a]	Gene no.[a]	Differential abundance[b]	Putative operon[c]
Terminal oxidation of alkanes						
14M	6.4	98.1	Regulator of the *alkB1GHJ* operon (AlkS)	ABO_2706	H	1
1M	6.5	46.5	Alkane 1-monooxygenase (AlkB)	ABO_2707	H	1
4M	4.5	18.7	Rubredoxin (AlkG)	ABO_2708	H	1
15M	9.5	52.8	Aldehyde dehydrogenase (AlkH)	ABO_2709	H	1
16M	9.3	58.3	Alcohol dehydrogenase (AlkJ)	ABO_2710	H	1
77M	9.5	57.3	Monooxygenase (putative)	ABO_0190	H	2
78M	8.4	60.6	Alcohol dehydrogenase (AlkJ-2)	ABO_0202	H	3
25C	6.2	53.5	Cytochrome P450-1	ABO_0201	[d]	3
25C	6.2	53.5	Cytochrome P450-2	ABO_2288	[d]	
Fatty acid oxidation						
23M	4.6	56.3	Long-chain fatty acid CoA ligase (FadB)	ABO_0184	H	4
15C	5.4	62.1	Long-chain fatty acid CoA ligase, putative (FadD)	ABO_0367	2.7 down	5
17M, 51M	5.0	59.8	Medium-chain fatty acid CoA ligase (AlkK)	ABO_2748	H	
22C	4.8	64.5	Acyl-CoA dehydrogenase (putative)	ABO_2102	H	
23C	5.6	42.5	Acyl-CoA dehydrogenase	ABO_0988	7.2 up	6
26M	5.3	77.3	Fatty acid oxidation complex (FadB2)	ABO_1652	H	7
12C, 25M	5.5	78.0	Fatty oxidation complex alpha subunit (FadB)	ABO_1566	61 up	8
Fatty acid and phospholipid biosynthesis						
13C	5.2	35.7	Acetyl-CoA carboxylase, carboxyl transferase, alpha subunit (AccA)	ABO_1159	2.2 down	
14C	5.6	48.9	Acetyl-CoA carboxylase, biotin carboxylase (AccC)	ABO_2010	1.9 down	9
34M	5.0	42.7	3-Oxoacyl-[acyl-carrier-protein] synthase (FabB)	ABO_0834	H	10
53M	5.2	41.3	3-Oxoacyl-[acyl-carrier-protein] synthase (FabB)	ABO_1520	6.4 up	
54M	4.9	42.1	3-oxoacyl-(acyl-carrier-protein) synthase (FabF)	ABO_1071	10.0 down	11
42M, 44M	9.6	45.3	Fatty acid desaturase (putative)	ABO_2585	H	
52M	8.9	36.4	Sterol desaturase family protein	ABO_0114	2.3 down	
30M	9.1	54.1	Cardiolipin synthase (Cls)	ABO_1816	H	
Amino acid biosynthesis						
75M	4.9	58.5	Dihydroxy-acid dehydratase (IlvD-1)	ABO_0180	H	4
82M	5.2	70.9	Dihydroxy-acid dehydratase (IlvD-2)	ABO_2312	P	
TCA, respiratory chain, glyoxylate bypass, and gluconeogenesis						
11C	5.5	82.6	Isocitrate dehydrogenase, NADH dependent (Icd)	ABO_1281	2.0 down	12
74M	4.8	51.2	2-Oxoglutarate dehydrogenase (LpdG)	ABO_1494	P	13
55M	5.1	32.0	Hydrolase	ABO_1541	P	14
9C	4.9	59.1	Isocitrate lyase (AceA)	ABO_2741	36 up	
10C	4.9	78.7	Malate synthase (GlcB)	ABO_1267	6.1 up	
6C	4.7	86.9	Phosphoenolpyruvate synthase (PpsA-1)	ABO_1427	H	
8C	5.0	45.8	Malic enzyme (MaeB)	ABO_2239	3.1 up	15
22M	8.8	13.2	Succinate dehydrogenase, hydrophobic membrane anchor protein (SdhD)	ABO_1499	H	16
76M	7.8	59.0	Oxidoreductase, GMC family	ABO_0187	5.6 up	4
Polyhydroxyalkanoate production (45M)	9.7	41.9	Poly-beta-hydroxybutyrate polymerase (PhaC)	ABO_1418	H	
Osmoprotection						
31M	4.8	14.8	Ectoin synthase (EctC)	ABO_2152	P	17
7C	5.0	48.4	DABA aminotransferase (EctB)	ABO_2151	P	17
Cofactor synthesis						
27M	8.7	40.4	(*S*)-2-Hydroxy fatty acid dehydrogenase (RibD)	ABO_2174	H	18
38M	6.2	27.3	Lipoil-(acyl-carrier protein)-protein-*n*-lipoyltrafsferase (LipB)	ABO_1963	26 down	19
Pilus formation (5C)	4.4	78.1	Fimbrial assembly protein precursor (PilQ)	ABO_2233	46 down	20

Continued on following page

TABLE 1—*Continued*

Spot no.	pI	Mol wt	Gene function or functional category[a]	Gene no.[a]	Differential abundance[b]	Putative operon[c]
Information processing and regulation						
21C	6.3	28.2	30S Ribosomal protein S2 (RpsB)	ABO_1143	H	21
16C	4.9	23.7	50S Ribosomal protein L25 (RplY)	ABO_0517	H	22
79M	6.5	91.6	Sensor histidine kinase	ABO_0442	14.0 up	
Transport proteins						
6M, 83M	9.0	87.1	ABC transporter, permease protein (putative)	ABO_1402	H	23
21M	4.8	47.1	ABC export system, membrane fusion protein	ABO_0248	7.2 up	24
63M	4.3	49.7	ABC export system, outer membrane protein	ABO_0250	H	24
81M	7.6	41.0	ABC transporter, ATP-binding protein, permease (putative)	ABO_1847	P	25
84M	6.5	67.6	Oligopeptide ABC transporter, periplasmic peptide-binding protein	ABO_1219	5.2 down	26
88M	8.6	69.6	Oligopeptide ABC transporter, periplasmic peptide-binding protein	ABO_1220	P	26
19C, 20C	9.2	37.3	Phosphate ABC transporter periplasmic binding protein (PstS)	ABO_2685	P	27
85M	8.7	44.3	Phosphate transporter (putative)	ABO_2305	P	
89M	5.9	56.4	Sodium solute transporter family protein	ABO_1913	H	
87M	9.7	95.9	Nitrite extrusion protein (NarK)	ABO_0547	H	28
49M	5.8	46.8	Metabolite transport transmembrane protein (putative)	ABO_2038	H	
24M	4.0	44.5	Long-chain fatty acid transporter (putative)	ABO_0572	20 up	
69M	5.0	62.5	Heavy metal RND efflux membrane fusion protein, CzcB family (CzcB2)	ABO_1357	3.1 up	29
62M	6.2	48.1	Heavy metal RND efflux outer membrane protein, CzcC family (CzcB1)	ABO_1358	P	29
59M	6.5	44.5	Heavy metal RND efflux membrane fusion protein, CZsB family (CzcB3)	ABO_1382	4.1 down	30
39M	4.6	40.7	Outer membrane polysaccharide export protein precursor (Wza)	ABO_0905	4.2 down	31
41M	3.9	36.1	Outer membrane porin (putative)	ABO_1621	P	
4C, 48M	4.4	80.2	FecA-like outer membrane receptor (FecA)	ABO_0721	43 down 2.5 up	
56M	4.4	14.1	Ferric siderophore transport system, inner membrane protein E (ExbD2)	ABO_1968	P	32
46M	4.2	23.0	Outer membrane lipoprotein carrier protein (LolA)	ABO_1291	P	33
2M	8.8	21.8	Outer membrane lipoprotein (LolB)	ABO_0520	H	34
29M	8.6	45.6	Lipoprotein-releasing system, permease protein (putative)	ABO_1049	H	35
67M	5.6	47.8	Multidrug/solvent RND membrane fusion protein (putative)	ABO_0965	H	36
68M	4.3	46.5	Membrane-associated zinc metalloprotease (putative)	ABO_1150	H	37

[a] Gene numbers, gene function, and functional category are presented according to the annotated genome (Schneiker et al., unpublished).
[b] H means that the protein is solely expressed on hexadecane; P means that the protein is solely expressed on pyruvate; "down" means that the protein is down expressed on hexadecane; "up" means the protein is up expressed on hexadecane.
[c] Putative operons demonstrated in this table are at least two consecutive genes encoding coexpressed functionally related proteins, closely associated on the chromosome and transcribed from their own putative promoter, identified by in silico analysis.
[d] For the expression pattern of the P450 cytochromes, refer to the text. TM, transmembrane domains (based on the TMHMM [30], a transmembrane helix prediction method based on a hidden Markov model).

89M). Sixteen proteins were detected exclusively in pyruvate-grown cells, namely, ectoin synthase EctC (spot 31M), outer membrane lipoprotein (spot 33M), inner membrane protein AmpE (spot 35M), putative membrane protein (spot 36M), putative outer membrane porin (spot 41M), outer membrane lipoprotein carrier protein LolA (spot 46M), ferric siderophore transport protein ExbD2 (spot 56M), hydrolase (spot 55M), membrane proteins (spots 61M and 65M), heavy metal RND efflux outer membrane protein CzcC (spot 62M), 2-oxoglutarate dehydrogenase LpdG (spot 74M), ABC transporter (spot 81M), dihydroxy-acid dehydratase IlvD2 (spot 82M), phosphate transporter (spot 85M), and oligopeptide ABC transporter (spot 88M). Identical MALDI spectra were obtained for the twin spots 19/20.

The combination of proteomic information obtained from the membrane and cytoplasmic fractions has yielded a rather comprehensive overview of the metabolic features of alkane-grown *Alcanivorax*, since enzymes involved in the primary attack of alkanes are mostly membrane bound (32, 36, 40, 48), whereas subsequent metabolism of the metabolic products occurs in the cytoplasm. Differentially expressed proteins fell into a number of groups of functionally related proteins, which

TABLE 2. Differentially expressed proteins of uncertain or unknown function

Spot no.	pI	Mol wt	Gene function or functional category[a]	Gene no.[a]	Differential abundance[b]	Putative operon[c]	Putative function based on sequence analysis tools
20M	4.9	17.3	Putative membrane protein	ABO_0097	31 down		Membrane protein implicated in regulation of membrane protease activity.
18C	6.0	49.7	LysM domain protein	ABO_0132	4.1 down		Lysin domain, found in enzymes involved in bacterial cell wall degradation.
28M	7.1	42.2	Hypothetical protein	ABO_0154	2.8 down		No putative domains have been detected.
3M	9.4	23.7	Hypothetical protein	ABO_0160	H		No putative domains have been detected.
3C	4.2	48.7	Hypothetical protein	ABO_0193	2.7 down	38	Similar to long-chain fatty acid transport protein of *Marinobacter aquaeolei* VT8.
33M	4.9	15.2	Outer membrane lipoprotein (OmlA)	ABO_0308	P		OmlA gene encodes a novel lipoprotein in *P. aeruginosa*. As in *Pseudomonas*, the gene *omlA* is immediately upstream of divergently transcribed *fur* (ferric uptake regulator).
64M	3.7	19.9	Membrane protein (putative)	ABO_0443	H		OmpA-like transmembrane domain is present in a number of different outer membrane proteins of several gram-negative bacteria.
73M	4.8	69.4	Lipoprotein (putative)	ABO_0586	H	39	Homologous to the LppC gene. A part of a putative operon with *gmhA* (ABO_556) encoding phosphoheptose isomerase, required for lipooligosaccharide biosynthesis in other bacteria (3).
35M	6.2	31.9	Inner membrane protein (AmpE)	ABO_0621	P	40	The AmpE gene encodes a transmembrane protein with unknown function. Upstream-located AmpD gene (ABO_587) encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase that participates in the intracellular recycling of peptidoglycan fragments.
65M	5.9	87.7	Membrane protein (putative)	ABO_0666	P	41	Predicted exporter of the RND superfamily.
40M, 2C	4.1	42.0	Outer membrane protein (OprF)	ABO_0822	55 down H		
61M	9.2	63.1	Membrane protein	ABO_0929	P	42	Putative sodium-sulphate transporter. TM.
66M	4.6	51.2	Membrane protein (putative)	ABO_0963	15.4 up	36	Outer membrane efflux protein. Forms trimeric channels that allow export of a variety of substrates in gram-negative bacteria.
7M	3.7	84.8	Conserved hypothetical protein	ABO_0997	H		No putative domains have been detected. TM.
47M	4.9	84.6	Membrane protein (putative)	ABO_1242	12 up		His kinase A (phosphoacceptor) domain.
36M	5.5	29.3	Membrane protein (putative)	ABO_1323	P	43	PEP-sugar phosphotransferase system, putatively involved in uptake of pyruvate in *Alcanivorax*. A part of a putative operon with the upstream gene (ABO_1249) encoding conserved hypothetical protein with pyruvate phosphate dikinase domain.
5M	8.5	101.8	Conserved hypothetical protein	ABO_1398	H		No putative domains have been detected. TM.
8M	3.5	108.8	Conserved hypothetical protein	ABO_1464	H	44	Tfp pilus assembly protein FimV. A part of an operon with the downstream *truA* gene in *P. aeruginosa* (1). The *fimV* gene is required for twitching motility while the *truA* gene is required for the type III secretory gene expression.
9M	4.8	105.3	Conserved hypothetical protein	ABO_1589	H	45	Family of proteins of unknown function.
32M	6.9	28.1	Conserved hypothetical protein	ABO_1588	H	45	Predicted spermidine synthase with an N-terminal membrane domain.
1C	3.9	36.1	Outer membrane protein	ABO_1621	P		Porins.
24C	5.9	41.8	Hypothetical protein	ABO_1657	4.1 down	46	No putative domains have been detected.
12M	4.5	95.7	Conserved hypothetical protein	ABO_1823	4.1 down	47	No putative domains have been detected. A part of a putative operon with the downstream genes. TM.
13M	3.7	24.3	Outer membrane protein (OprG)	ABO_1922	H		A major outer membrane protein of *P. aeruginosa* (16); closest homology to *Vibrio cholerae ompW*; is probably involved in low-affinity iron uptake (52).
57M	6.7	23.3	Membrane protein (putative)	ABO_1971	3.7 down		Predicted divalent heavy metal cation transporter.
10M	7.0	86.1	Conserved hypothetical protein	ABO_2083	12.0 up		Ankyrin repeats; ankyrin repeats mediate protein-protein interactions in very diverse families of proteins.
70M	5.2	39.8	Outer membrane phospholipase A precursor	ABO_2104	13 down		Outer membrane phospholipase A is an outer membrane-localized enzyme. It is implicated in the virulence of several pathogens.
17C	4.4	43.8	Conserved hypothetical protein	ABO_2153	4.6 down		No putative domains have been detected.
72M	6.4	58.5	Membrane protein (putative)	ABO_2547	18 down		Uncharacterized iron-regulated membrane protein. TM.
60M	4.8	65.1	Inner membrane protein	ABO_2753	4.3 down	48	This family of proteins is required for the insertion of integral membrane proteins into cellular membranes. Can be associated with respiratory chain complexes.

[a] Gene numbers, gene function, and functional category are presented according to the annotated genome (Schneiker et al., unpublished).

[b] H means that the protein is solely expressed on hexadecane; P means that the protein is solely expressed on pyruvate; "down" means that the protein is down-expressed on hexadecane; "up" means the protein is up-expressed on hexadecane.

[c] Putative operons demonstrated in this table are at least two consecutive genes encoding coexpressed functionally related proteins, closely associated on the chromosome and transcribed from their own putative promoter, identified by in silico analysis.

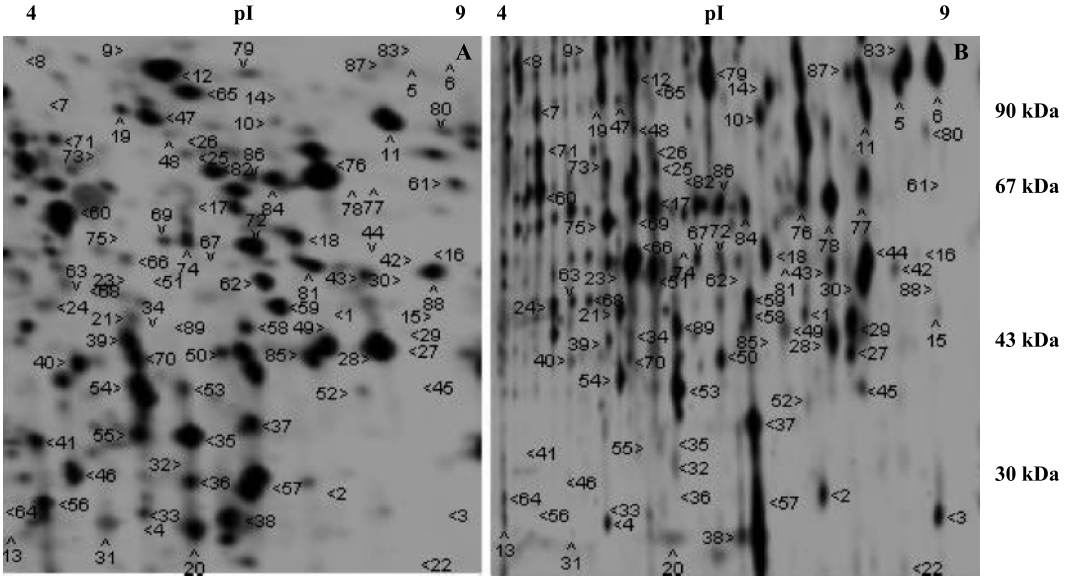

FIG. 2. A 2-D map of the membrane proteins of *Alcanivorax borkumensis* SK2. Cells were grown on either pyruvate (A) or hexadecane (B). Isoelectric focusing was performed using an IPG strip of pH 4 to 9. All membrane proteins identified are numbered.

often led to the formation of distinct gene clusters on the chromosome. These clusters were found to represent or include some 46 putative operons, as defined by common orientation of genes, a maximum of 50 bp between successive genes (17), and the presence of putative promoters. Many of the membrane proteins up-regulated by growth on alkanes appeared to be related to metabolic pathways directly involved or closely linked to the metabolism of alkanes, namely, the terminal oxidation of alkanes, fatty acid oxidation, and polyhydroxyalkanoate production, the latter representing a major pathway for carbon storage under conditions of excess carbon supply (J. S. Sabirova et al., unpublished data). Cytoplasmic responses to growth on alkanes were found to mostly concern the activity of intracellular carbon fluxes (glyoxylate bypass, fatty acid synthesis, and fatty acid oxidation). In addition, we found a number of differentially expressed conserved hypothetical proteins and membrane proteins of unknown function, the potential functions of which are listed in Table 2, as far as they could be identified by the means of various sequence analysis tools.

In the following paragraphs, we analyze the principal metabolic routes that adapted to growth on alkanes.

Terminal alkane oxidation. Aerobic metabolism of alkanes generally proceeds through sequential oxidation of a terminal carbon, initiated by monooxygenases, which produce the alcohols, and followed by alcohol and aldehyde dehydrogenases, which produce the corresponding aldehydes and fatty acids, respectively. As indicated above, several monooxygenases and enzymes catalyzing subsequent oxidations were found uniquely in the membrane fraction of hexadecane-grown cells (Table 1). Among these was the entire set of enzymes of the *alkB1* operon, consisting of ABO_2707 encoding the AlkB1 alkane monooxygenase, ABO_2708 encoding the AlkG rubredoxin, ABO_2709 encoding the AlkH aldehyde dehydrogenase, and ABO_2710 encoding the AlkJ alcohol dehydrogenase (Fig. 3A), described by van Beilen et al. (46) for *A. borkumensis* strain AP1. We also detected alkane-induced expression of the regulator AlkS (ABO_2706), whose gene lies upstream of, and is divergently oriented from, the *alkB1* operon and which has been described as the transcriptional activator of the *alkB* cluster in *Pseudomonas oleovorans* (11).

Other enzymes found to be alkane-induced were cytochrome P450 monooxygenase encoded by ABO_0201 (and/or ABO_2288; see below) and AlkJ2 alcohol dehydrogenase encoded by ABO_0202, which form part of a putative operon comprising the genes encoding ferredoxin (ABO_0200), cytochrome P450-1 (ABO_0201), AlkJ2-alcohol dehydrogenase (ABO_0202), and an oxidoreductase (ABO_0203) (Fig. 3C).

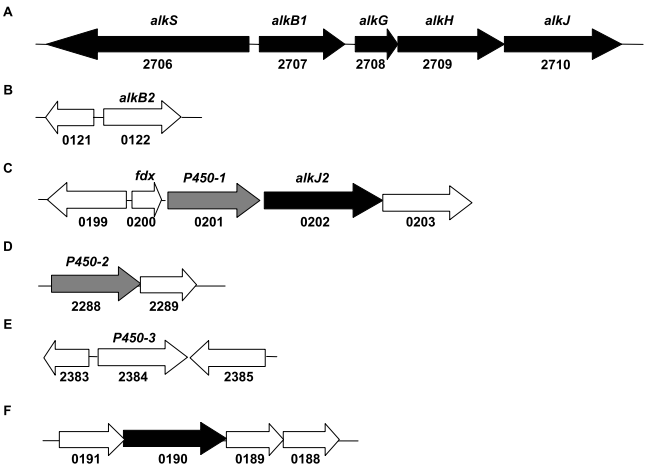

FIG. 3. Schematic representation of genomic regions containing genes encoding monooxygenases and enzymes presumed to be involved in terminal oxidation of alkanes. Genes up-regulated in alkane-grown cells are in black; white-colored open reading frames with black frames show homologous genes in the *A. borkumensis* genome that seem not to be up-regulated; the gray-colored open reading frames encode the P450-1 and P450-2 enzymes.

The P450-1 putative operon is closely linked to an AraC-like transcriptional regulator (ABO_0199) reading in the opposite direction. The amino acid sequence of the *alkJ2*-encoded alcohol dehydrogenase (ABO_0202) shows strong homology to at least two other *A. borkumensis* alcohol dehydrogenases, one of which is AlkJ (ABO_2710) of the *alkB1* operon. P450-1 cytochrome encoded by ABO_0201 is identical to a second P450 cytochrome, P450-2, encoded by ABO_2288 (Fig. 3D), and also strongly homologous to a third P450 cytochrome, P450-3, encoded by ABO_2384 (Fig. 3E). P450 cytochromes belong to a superfamily of heme proteins found in all eukaryotes, as well as in most prokaryotes and archaea (35), which catalyze monooxygenation of a wide variety of organic molecules. The involvement of P450 enzymes in alkane degradation has previously been shown for *Alcanivorax borkumensis* AP1, *Rhodococcus rhodochrous* ATCC 19067, *Acinetobacter calcoaceticus* EB 104, a *Corynebacterium* sp., and some hydrocarbon-degrading yeasts (4, 33, 30, 45). Figure 1 revealed expression of either P450-1 and/or P450-2 in cells grown on either alkane or pyruvate but at higher levels in alkane-grown cells. P450-1, although not apparent on 2-D gels, is presumably up-regulated in cells grown on alkanes, since its gene is in the same operon as that of the up-regulated *alkJ2* gene, but this is probably not the case for P450-2 and P450-3. In silico comparison of the regions upstream of ABO_0201 (P450-1) and ABO_2288 (P450-2) to promoter consensus sequences of other bacteria revealed that these two genes are preceded by different putative promoter sequences and are thus likely to be differently regulated (Fig. 3C and D). While ABO_0201 is likely to be induced by alkanes, ABO_2288 is probably constitutively expressed. It is not yet clear which role, other than a factor in the primary attack of alkanes, P450 cytochromes may play in *Alcanivorax*, such as in cells growing on pyruvate. However, there is evidence that P450 in *Bacillus subtilis* is involved in supplying pimelic acid equivalents for the synthesis of biotin (8), a cofactor of the principal enzymes of fatty acid biosynthesis.

We have also detected alkane-induced expression of a putative monooxygenase encoded by ABO_0190 (Fig. 3F). In silico analysis (Pfam) identified this monooxygenase as a flavin-binding monooxygenase belonging to a family of xenobiotic-metabolizing enzymes and with 52% identity/68% similarity to cyclohexanone monooxygenase of *Ralstonia eutropha* strain JMP134, an enzyme that mediates oxidation of cyclohexanone, the second step in the metabolism of cyclohexane. Inspection of the *Alcanivorax* genomic context of ABO_0190 revealed a putative operon of four genes encoding caprolactone hydrolase (ABO_0191), cyclohexanone monooxygenase (ABO_0190), cyclohexanol dehydrogenase (ABO_0189), and metal-dependent hydrolase (ABO_0188) that are probably involved in the metabolism of cycloalkanes and convert cyclohexanol to 6-hydroxyhexanoic acid. We did not find a gene encoding cyclohexane monooxygenase in the *A. borkumensis* genome and therefore suspect that another enzyme mediates the initial attack of cyclic alkanes in *Alcanivorax* and that this enzyme may in fact be alkane hydroxylase, encoded by either *alkB1* or *alkB2*, since Fujii et al. (15) recently showed oxidation of cycloalkanes by the alkane hydroxylase system (comprising alkane 1-monooxygenase AlkB, rubredoxin AlkG, and rubredoxin AlkT) of *Gordonia* sp. TF6. The assumption that initial oxidation of linear alkanes and cyclohexanes by *A. borkumensis* is mediated by the same AlkB1/AlkB2 monooxygenase is consistent with (i) the inability of *A. borkumensis* to utilize cyclohexane as the sole source of carbon and energy (data not shown) but the ability to cometabolize it well when grown on crude oil (M. M. Yakimov et al., unpublished data) and (ii) the apparent coinduction by hexadecane of both AlkB1 and the predicted cyclohexane-degrading operon containing the up-regulated putative cyclohexanone monooxygenase-encoding ABO_0190 (also reflected in some marked similarities of the respective operon upstream regions which both exhibit putative σ^{70} promoters as well as some perfectly conserved sequence motives of a likely regulatory function) (data not shown). These tentative conclusions await experimental confirmation.

Fatty acids, lipids, and membranes. Fatty acids produced during growth on alkanes are transformed into CoA-activated fatty acids, which are further degraded via β-oxidation. As was expected, alkane degradation by *A. borkumensis* is associated with increased expression of enzymes of the β-oxidation pathway. Interestingly, two entire sets of β-oxidation enzymes were induced by growth on alkanes, namely, ABO_0184 and ABO_2748, which encode fatty acid CoA ligases (synthetases), ABO_2102 and ABO_0988, which encode acyl-CoA dehydrogenases, and ABO_1652 and ABO_1566, which encode bifunctional components of the β-oxidation multifunctional enzyme complex and that possess both 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities. ABO_1652 and ABO_1566 encode components of two different enzyme complexes involved in alkane-induced fatty acid oxidation, one of which, ABO_1652, corresponding to *fadB2* of the *fadAB2* operon, is exclusively expressed in cells grown on hexadecane, while the latter, ABO_1566, corresponding to *fadB* of the *fadAB* operon, though clearly up-regulated in presence of alkanes, is also expressed in cells grown on pyruvate.

Apart from enhanced expression of genes mediating fatty acid degradation, we also detected a significant alkane-induced increase of expression of fatty acid biosynthesis determinants, namely, the *fabAB* operon ABO_0835 and ABO_0834, encoding β-hydroxyacyl-acyl carrier protein dehydratase (FabA) and β-ketoacyl-acyl carrier protein synthase I (FabB), and ABO_1520, which encodes a second FabB homologue. The Fab enzymes of *Escherichia coli* (7, 39) and *Pseudomonas aeruginosa* (23) have been shown to be specifically required for the synthesis of unsaturated fatty acids. It is known that growth of *Pseudomonas oleovorans* GPo1 on alkanes results in substantial accumulation of AlkB alkane hydroxylase protein in the inner membrane (12), which would provoke perturbation of membrane structure and physical characteristics if not compensated for by an increase in lipids with unsaturated fatty acids (5). The up-regulation of enzymes involved in the synthesis of unsaturated fatty acids is presumably associated with the need to maintain membrane fluidity and integrity in step with increasing AlkB protein-induced perturbations.

In contrast, we observed alkane-induced down-regulation of cytoplasmic proteins acetyl-CoA carboxylase AccA, encoded by ABO_1159, and acetyl-CoA carboxylase AccC, encoded by ABO_2010, components of a multicomponent system catalyzing the first step in the synthesis of fatty acids, namely, the production of malonyl-CoA from acetyl-CoA, also involved in

fatty acid biosynthesis. This is consistent with a reduced requirement for malate in alkane-grown cells, which is produced in abundance as a result of increased activity of the glyoxylate bypass (see below).

Alkane-induced changes in the composition of the cellular fatty acid pool are indicative of concomitant changes in membrane lipid composition (and indeed, membrane composition). In this regard, we observed increased expression of ABO_1816, which encodes cardiolipin synthase (Cls) in alkane-grown cells. Cardiolipin has been shown to have the potential to form nonbilayer structures, which introduce discontinuities into lipid bilayers, and thus to facilitate dynamic changes in membrane structures, such as membrane fusion events (e.g., the formation of adhesion sites between the outer and inner membranes, during cell division) (10), but also to activate membrane-bound enzymes, like AlkB (25, 34). Importantly, increased cardiolipin synthesis may also constitute a protective membrane adaptation to decrease membrane permeability to organic solvents, as has been shown for pseudomonads (47).

Another alkane-induced change related to lipid metabolism that we observed was the up-regulation of genes coding for the lipoprotein-releasing proteins (Lol proteins), which target and anchor lipoproteins to the periplasmic surface of either the inner or the outer membrane, depending on the sorting signal (44). The Lol system consists of an ATP-binding cassette transporter, encoded by *lolCDE*, which transports outer membrane-specific lipoproteins across the inner membrane into the periplasmic space, where they are released. An intermembrane shuttle complex then forms between the released lipoproteins and the LolA periplasmic chaperone, which then associates with the LolB outer membrane-located permease to complete the targeting process (44). Expression of ABO_1049 and ABO_1050, encoding the LolCDE transporter, and ABO_0520, encoding the LolB permease, was up-regulated in alkane-grown cells. Surprisingly, expression of ABO_1291, which encodes the LolA periplasmic chaperone, was detected only in cells grown on pyruvate. This finding may, however, be misleading, as the LolA chaperone may be tightly complexed with its target lipoproteins and thus in a form not readily resolved by proteomics. Alkane-induced expression of the Lol system in *Alcanivorax* might reflect an increased need to release lipoproteins, since some of these have been shown to possess emulsifying properties that increase the surface area and hence enhance the bioavailability of hydrophobic substrates (29, 31, 51).

Glyoxylate bypass and gluconeogenesis. During growth on alkanes as the sole carbon source, bacteria must generate all cellular precursor metabolites from acetyl-CoA, the main intermediate formed during alkane degradation via β-oxidation of fatty acids. One mechanism to do this is the short circuiting of the citric acid cycle, through activation of the glyoxylate bypass, which routes acetyl-CoA to the key 3-carbon metabolite phosphoenolpyruvate, via isocitrate, glyoxylate, and malate, by means of isocitrate lyase and malate synthase, thereby avoiding the CO_2-releasing steps of the cycle (Fig. 4). A significant feature of alkane-grown cells is the up-regulation of ABO_2741, encoding isocitrate lyase AceA, and ABO_1267, encoding malate synthase GlcB, and down-regulation of enzymes mediating CO_2-releasing steps of the tricarboxylic acid (TCA) cycle short circuited by the glyoxylate shunt, including ABO_1281, encoding isocitrate dehydrogenase Icd, and ABO_1494,

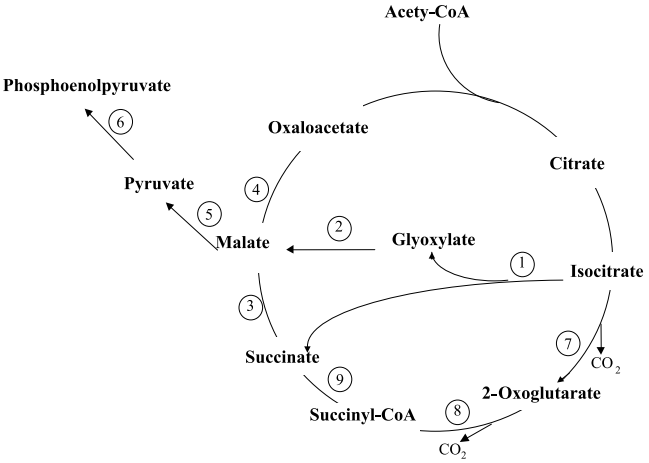

FIG. 4. Increased exploitation of the glyoxylate bypass of the TCA cycle in alkane-grown cells of *Alcanivorax*. The glyoxylate bypass is carried out by isocitrate lyase (1) and malate synthase (2). Succinate produced via glyoxylate bypass is converted to malate by succinate dehydrogenase (3). Malate is converted to oxaloacetate by malate dehydrogenase (4) or is used by malic enzyme (5) in gluconeogenesis to produce pyruvate. Pyruvate is then converted by phosphoenolpyruvate synthase (6) to produce phosphoenolpyruvate. The incomplete TCA cycle is associated with the alkane-induced down-regulation of isocitrate dehydrogenase (7), 2-oxoglutarate dehydrogenase (8), and succinyl-CoA synthetase (9).

encoding 2-oxoglutarate dehydrogenase LpdG. We also suspect down-regulation of another enzyme indicative of a complete TCA cycle, namely, succinyl-CoA synthetase SucC, encoded by ABO_1493, as according to our in silico analysis, this gene is located in the same putative operon as ABO_1494. Another enzyme of the TCA cycle needed for the glyoxylate bypass, namely, succinate dehydrogenase SdhD (ABO_1499), was also found to be up-regulated in alkane-grown cells (Fig. 4).

The hypothesis that all biosynthetic precursors come from acetyl-CoA in alkane-grown cells is also consistent with the finding that enzymes involved in gluconeogenesis, namely, malic enzyme MaeB (ABO_2239) and phosphoenolpyruvate synthase PspA-1 (ABO_1427), were up-regulated (Fig. 4). Thus, the key metabolic intermediate in alkane-grown cells is malate, formed through the channeling of acetyl-CoA into the glyoxylate bypass.

Polyhydroxyalkanoate biosynthesis. For carbon-limited microbes, an increase in carbon allows an increase in growth rate until another growth limitation is reached. The appearance of alkanes in oligotrophic environments like most marine habitats allows *Alcanivorax* to "bloom" initially until nitrogen limitation is experienced. Under conditions of high C/N ratios, many microbes synthesize carbon storage materials, like polyhydroxyalkanoates (PHAs). ABO_1418, one of two *A. borkumensis phaC* PHA synthase genes, was solely expressed in alkane-grown cells, whereas another, ABO_2214, was not expressed at detectable levels in such cells. Since PHA is also produced at high C/N ratios in cells grown on nonalkane substrates (Sabirova et al., unpublished), it seems that *A. borkumensis* produces one of two distinct PhaC PHA synthases in response to different growth substrates, probably having different substrate

specificities that reflect distinct metabolites produced from the different growth substrates.

Cofactor synthesis. Alkane metabolism in *Alcanivorax* involves a number of enzymes, in particular monooxygenases, containing cofactors as active groups. ABO_217, encoding RibD [(*S*)-2-hydroxy-fatty-acid dehydrogenase], a key enzyme of the riboflavin synthesis pathway, is up-regulated in alkane-grown cells. Riboflavin is the precursor of flavin mononucleotide and flavin adenine dinucleotide, cofactors of enzymes involved in reduction processes and of electron transport proteins, such as dehydrogenases, oxidases, and monooxygenases. Specifically, riboflavins are cofactors of the flavin-binding monooxygenase encoded by ABO_0190 and of the flavin mononucleotide-binding domains of P450 cytochromes encoded by ABO_0201 and ABO_2288. On the other hand, ABO_1963, encoding lipoil-(acyl-carrier protein)-protein-*n*-lipoyltransferase, an enzyme involved in biotin biosynthesis, is down-regulated in alkane-grown cells. Down-regulation of biotin biosynthesis is consistent with the alkane-induced repression of the *accA* and *accC* genes, encoding key enzymes of the fatty acid biosynthetic route, in which biotin serves as a cofactor.

Conclusions. The data presented here strongly suggest that alkane degradation in *Alcanivorax* proceeds via several routes of terminal oxidation, involving AlkB hydroxylases, a putative flavin-binding monooxygenase, and P450 cytochrome(s). Since certain individual *n*-alkanes and cycloalkanes are not growth substrates but are metabolized when present in hydrocarbon mixtures, they may not be inducers of the initial monooxygenases and require other alkanes to induce the appropriate catabolic enzymes. Alkane degradation strongly modifies metabolism, especially intracellular carbon fluxes and membrane lipid composition. The glyoxylate bypass and gluconeogenesis routes induced by alkanes adapt the cell to produce key cellular precursor metabolites directly from the fatty acids produced by alkane oxidation. Despite the fact that, as would be expected, fatty acid synthesis is down-regulated during growth on alkanes, there is an up-regulation of the synthesis of unsaturated fatty acids, presumably reflecting a need for resulting changes in the composition of membrane lipids. These results provide new insights into the metabolic adaptations needed for growth on alkanes and into the genomic basis of the hydrocarbonoclastic lifestyle. Since the 2-D maps have revealed a number of up-regulated proteins with unknown functions in alkane metabolism, their study will surely bring further important insights into this unique lifestyle.

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